A HISTOPHYSIOLOGICAL STUDY OF THE RAT DIAPHRAGM

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Though some extensive studies have been made on the general morphology and physiology of the mammalian diaphragm as a whole, sufficient attention has not been paid to the regional and cellular organization of this organ. Morphologically, three distinct regions, namely vertebral (dorsal), costal (lateral) and sternal (ventral) have been described in man (Johnston and Whillis, 1949). But little information is available on the cellular components which constitute these regions, with regard to their histological features and metabolic activities. In a recent study Frunder (1954) observed that the oxygen uptake in the posterior and anterior parts of the diaphragm differed with regard to their glycogen content, and pointed out possible errors in respiration studies with selected parts of the diaphragm.

Recent studies conducted in our laboratories on the structure and physiology of mixed muscles, such as the pigeon breast muscle, which have been reviewed by Drummond and Black (1960), have shown the existence of two types of fibers: one broad, white and glycogen-loaded, adapted for an anaerobic metabolism in which glycogen is the chief fuel for energy; and a narrow, red fat-loaded variety for aerobic metabolism in which fat forms the chief fuel. The diaphragm also being a mixed type of muscle, it was thought desirable to carry out similar studies on its different regions with a view to correlating structure with function. In our present investigation the three regions of the rat diaphragm have been studied with regard to the nature, number and distribution pattern, diameter, metabolite load and concentrations of enzymes (lipase and succinic dehydrogenase) of the constituent fibers.

Materials and Methods

Freshly collected wild rats (*Rattus norvegicus*) were used as the material in the present investigation. The rats were decapitated and the whole diaphragm was quickly removed and spread on a clean filter paper. The three regions were demarcated and cut out separately. For histochemical observations thin strips of the tissue were then cut in line with the orientation of the fibers. For the quantitative estimations, each separate region was pooled from a number of individuals.

Fiber count and fiber diameter

Thin, frozen, hand sections of all the three regions, were separately cut out, spread on clean, dry slides and mounted in glycerine jelly. The respective numbers of red and white fibers within a certain lens field were counted. With the aid of an ocular eyepiece and micrometer scale the diameter of the fibers was determined.

Fat: histochemical localization and quantitative estimation

The tissue was fixed in Baker's calcium formol (Baker, 1946) for 24 hours and washed in running tap water for the same time and embedded in 20% gelatin. Thirty- to forty-micron sections were cut on a freezing microtome and stained for fat, using the Sudan Black B stain.

As already mentioned the material from a single individual was found to be insufficient for quantitative estimations and so the material from a number of individuals, irrespective of sex, was pooled together. It was then dehydrated by drying in a hot air oven at 100° C. The total fat content was estimated by the Soxhlet extraction method, using a 1 : 1 alcohol-ether mixture.

Glycogen: histochemical localization and quantitative estimation

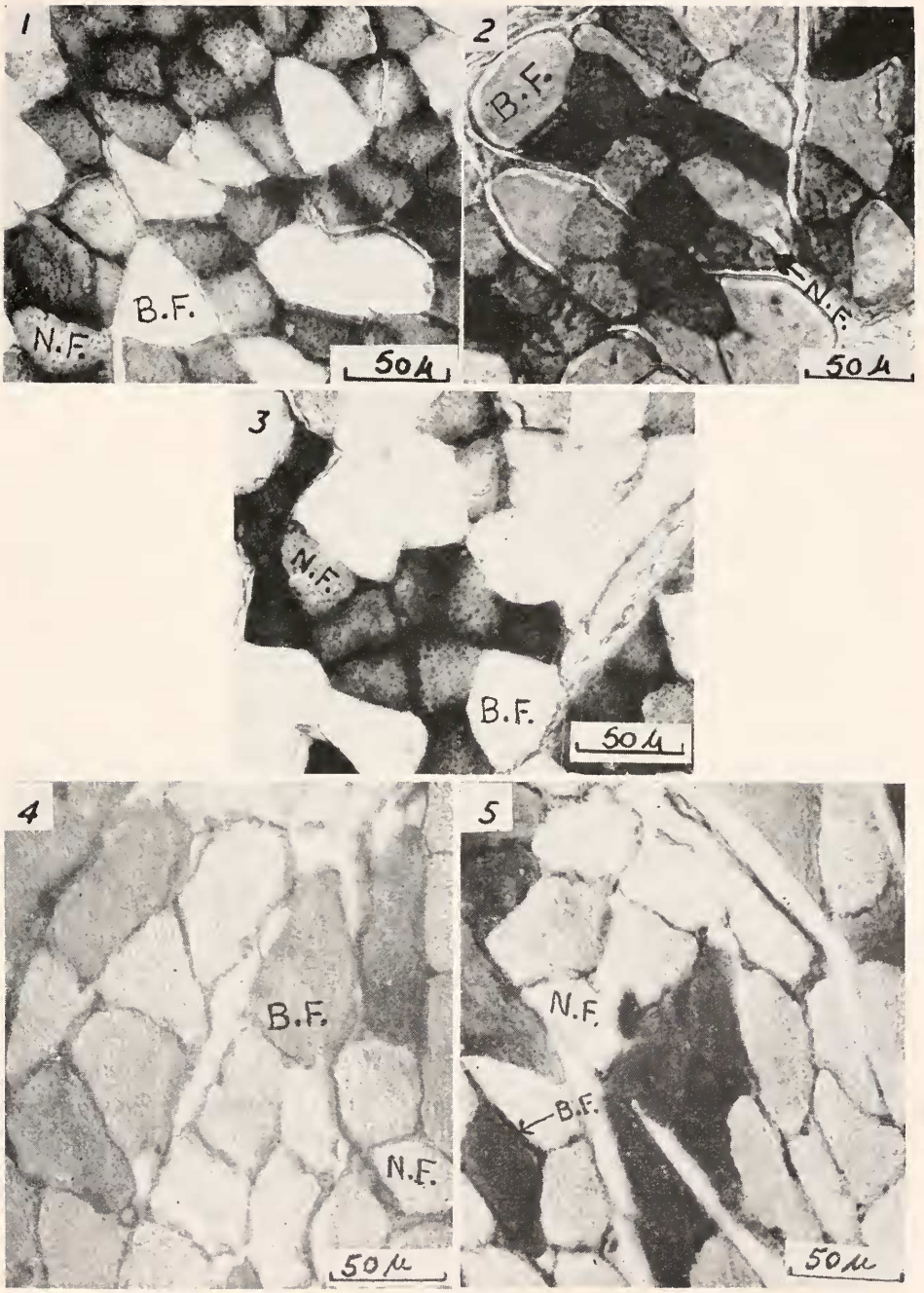
The tissue was fixed in cold alcoholic picroformol for 24 hours and embedded in paraffin wax of melting point 56 to 58° C. Sections 10 to 12 μ thick were cut and stained for glycogen by the periodic acid Schiff's reagent (Pearse, 1960).

Glycogen was estimated quantitatively by the micro method of Kemp *et al.* The material was pooled and treated with 80% methanol for the removal of glucose. The insoluble part was deproteinized and the clear supernatant hydrolyzed by concentrated sulphuric acid. The intensity of the pink color was measured on a Klett-Summerson photoelectric colorimeter, using a 520 mμ filter. The amount of glycogen present in the material was directly read from the standard graph. The results are expressed as μg. glycogen/100 mg. wet tissue.

Lipase: histochemical localization and quantitative estimation

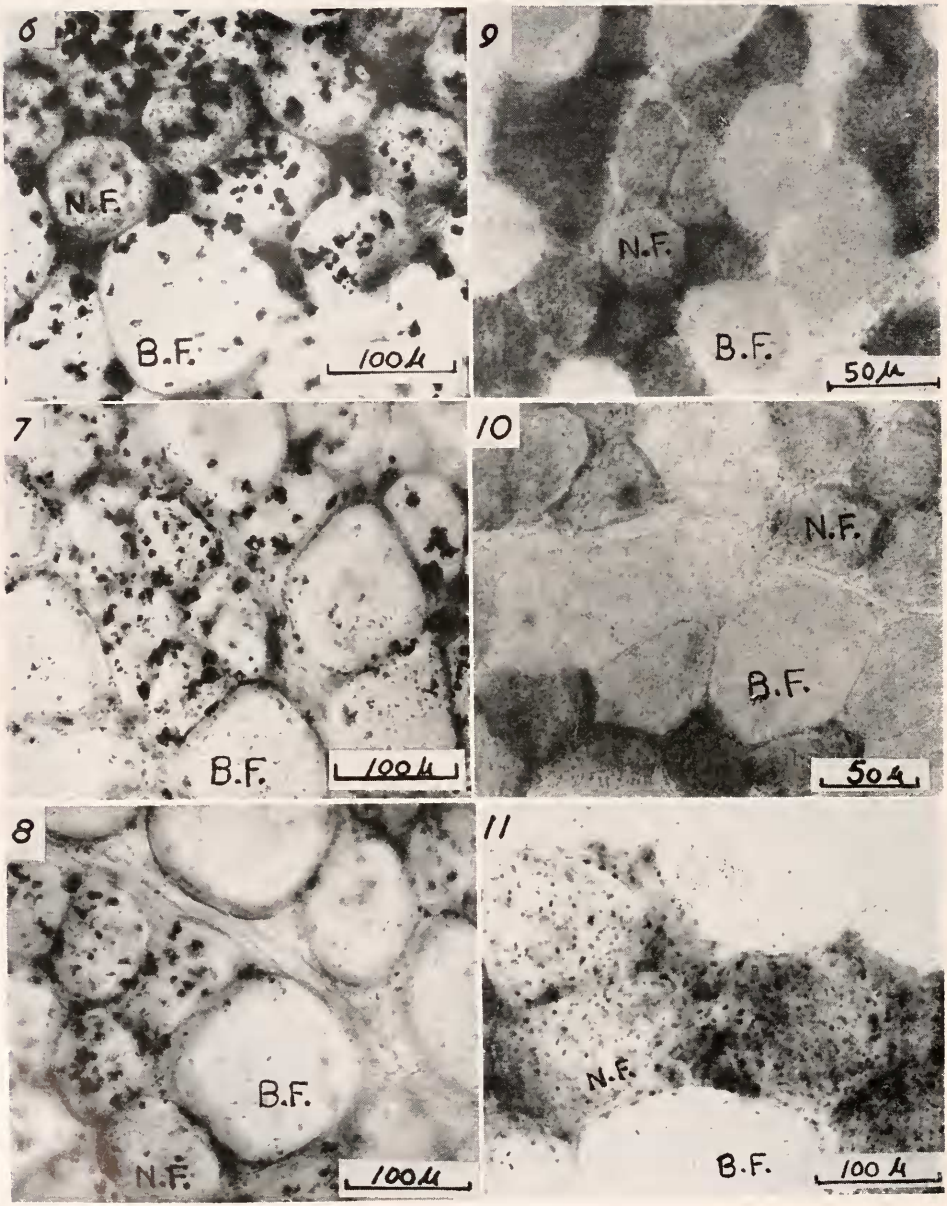
The lipase activity in the two types of fibers was studied histochemically using an improved technique (George and Iype, 1960). Thin frozen sections were cut according to the method of George and Scaria (1958) and dried on clean albumenized slides at room temperature. The sections were fixed in cold 6% neutral formalin for 3 to 4 hours, washed well in running tap water for about an hour and rinsed well with distilled water. A thin coating of gelatin (5%) was then applied on the sections and fixed in 6% neutral formalin for half an hour. It was washed in tap water for another half an hour. The sample sections were incubated for 12 to 16 hours in the incubation medium with "Tween 80" as substrate. The control sections were boiled for 10 minutes, coated with gelatin and incubated along with the sample sections. After incubation, the sections were washed well with distilled water and then with warm water (40° C.), so that the gelatin coating was completely removed. The sections were then treated with lead nitrate for half an hour and then with dilute yellow ammonium sulphide. According to the modifications made by George and Iype (1960), a final step of rinsing the sections in 1–2% acetic acid was also adopted for clearing the sections. The brownish black precipitates of lead sulphide formed as the result of the enzymic activity were seen very prominently.

The quantitative estimation of lipase activity was carried out according to the method of Martin and Peers (1953) using a Warburg manometric apparatus. The results obtained are presented as μl. CO_2 produced per mg. protein per hour.



FIGURES 1, 2, and 3. Transverse sections of the dorsal, ventral, and lateral regions, respectively, of the diaphragm stained with Sudan Black B for fat. The broad fibers (B.F.) are less sudanophilic than the narrow ones (N.F.).

FIGURES 4 and 5. Transverse sections of the dorsal and lateral regions, respectively, of the diaphragm stained with PAS for glycogen. The broad fibers contain more glycogen than the narrow ones.



FIGURES 6, 7 and 8. Histochemical localization of lipase activity in the fibers of the dorsal, ventral and lateral regions, respectively. The enzymic activity in the narrow fibers is more than in the broad ones.

FIGURES 9, 10 and 11. Histochemical demonstration of succinic dehydrogenase activity in the fibers of the dorsal, ventral and lateral regions. The deposition of the diformazan granules is more in the narrow fibers.

The protein content of the tissue was estimated by the micro-Kjeldahl steam distillation method (Hawk *et al.*, 1954).

Succinic dehydrogenase: histochemical localization and quantitative estimation

Thin frozen sections of the three regions of the diaphragm were separately cut out and the histochemical demonstration of succinic dehydrogenase was carried out by an improved method of George and Talesara (1961) using neotetrazolium chloride as the hydrogen acceptor. The sections were transferred to cold 0.1 *M* phosphate buffer (pH 7.4) for 10 minutes to destroy the endogenous substrates. They were then transferred to the incubation medium consisting of 0.1 *M* phosphate buffer (pH 7.4) 2.5 ml., 0.5 *M* sodium succinate 0.6 ml., 0.004 *M* aluminium chloride 0.5 ml., neotetrazolium chloride (3 mg. per ml.) 1 ml., methylene blue (1 mg. per ml.) 0.1 ml., 0.6 *M* sodium bicarbonate 0.3 ml. and 0.005 *M* magnesium sulphate one drop. The incubation was carried out under strictly anaerobic conditions by passing a mixture of nitrogen (95%) and carbon dioxide (5%) through the incubation medium at 37° C. for 5 to 10 minutes. The purple granules of the diformazan formed indicated the sites of the enzymic activity.

The enzyme activity was assessed quantitatively by the colorimetric method of Kun and Abood (1949). The results are expressed as μg. formazan formed per mg. dry weight per two hours.

Results

Histochemical

Fat. In all the three regions it was found that the broad fibers were less sudanophilic than the narrow ones (Figs. 1, 2, 3).

Glycogen. In the dorsal and lateral regions, the broad white fibers were found to contain more glycogen than the narrow ones (Figs. 4, 5). In the ventral region, however, the glycogen content was so low that satisfactory histochemical demonstration was not possible.

Lipase. The level of lipase activity in the three regions differed according to the nature and distribution of the fibers present, the broader fibers having less lipase activity (Figs. 6, 7, 8).

Table I

Giving the relative distribution, metabolite load and enzyme (lipase and succinic dehydrogenase) activity in the two types of fibers in the three regions of the rat diaphragm

Region of the diaphragm	Number of fibers in % per unit area		Ratio of the fibers B:N	Diameter of the fibers in μ		Diameter ratio B:N	Area covered per unit area in μ^2	
	Broad	Narrow		Broad	Narrow		Broad	Narrow
Dorsal (vertebral)	29.23	70.76	1:2.4	78.30	40.50	1:1.9	32,230	21,530
Lateral (costal)	32.29	67.70	1:2.1	90.72	43.20	1:2.1	40,100	19,060
Ventral (sternal)	32.24	67.78	1:2.1	49.40	37.80	1:1.3	18,890	23,020

TABLE I—(*Continued*)

Region of the diaphragm	Area ratio B:N	Glycogen μg./100 mg. wet tissue	Fat % dry weight	Lipase μl. CO_2/ mg. protein/ hour	SDH μg. formazan/ mg. dry weight/ 2 hours
		S.D.	S.D.	S.D.	S.D.
Dorsal (vertebral)	1:0.7	232.72 ± 24.95	16.19 ± 1.43	41.54 ± 5.52	10.97 ± 2.45
Lateral (costal)	1:0.5	297.42 ± 21.37	13.17 ± 1.55	24.55 ± 4.25	14.16 ± 3.22
Ventral (sternal)	1:1.2	127.42 ± 15.68	21.41 ± 0.80	29.79 ± 3.11	8.06 ± 2.81

Succinic dehydrogenase. The broad fibers showed considerably less enzyme activity than the narrow ones, in all the regions. In the lateral region, however, the narrow fibers, even though broader than the narrow fibers of the other two regions, showed high enzyme activity (Figs. 9, 10, 11).

Quantitative

The data obtained from the quantitative estimations are given in Table I. From Table I it is clear that each of the three regions has its own characteristics. The dorsal region has the highest number of narrow fibers and the least number of broad fibers, the diameter of the narrow fibers being less than that of the narrow fibers of the lateral region but higher than those of the ventral region. The diameter of the broad fibers is, however, less than that of the broad fibers of the lateral region but more than that of the broad fibers of the ventral. The area covered in a unit space by the broad fibers is less than that covered by the broad ones of the lateral and the area covered by the narrow fibers in the same space is less than that covered by the narrow ones of the ventral. The glycogen content is less than that in the lateral but more than that in the ventral. The fat content is higher than that in the lateral while it is less than that in the ventral. The dorsal region, again, has the highest lipase activity among all the three regions and succinic dehydrogenase activity less than that in the lateral but more than that in the ventral.

Of the three regions the ventral has the narrowest narrow fibers; narrowest broad fibers; the largest area covered by narrow fibers; has the least glycogen content; highest fat content; high lipase activity, much higher than the lateral but lower than in the dorsal, and the least succinic dehydrogenase activity.

The lateral region, on the other hand, possesses the broadest broad fibers; broadest narrow fibers; largest area covered by the broad fibers; highest glycogen load; least fat load; least lipase activity but highest succinic dehydrogenase activity.

DISCUSSION

That the diaphragm is a mixed type of muscle, having fibers of different sizes, is well known. Gunther (1953) studied the human, dog, and rat diaphragms and distinguished two types of muscle fibers, tetanic and tonic ones, the latter amounting to 10% in the human, 42% in the rat and 5% in the dog. He thus suggested a functional difference in the two types of fibers. Recently Nachmias and Padykula

(1958) in their histochemical study observed in the rat diaphragm two types of fibers, those with smaller average diameter having greater succinic dehydrogenase activity and others with larger diameter with lesser enzymic activity. In the cat diaphragm, however, they described three types of fibers, the third type being characterized by intense uptake of Sudan Black B in the subsarcolemmal position.

In the present study, two distinct types of fibers, namely a broad white and a narrow red, similar to the two types described in the pigeon breast muscle (George and Naik, 1957), existing side by side in all the three regions of the diaphragm, have been recognized. However, the distribution pattern of these two types of fibers, in contrast to that of the pigeon breast muscle (George and Naik, 1959), does not show any definite design and appears to be at random. It was also observed that the diameter of the fibers, unlike that of the pigeon breast muscle, showed a high degree of variation in each of the two types. Nevertheless, from our histochemical observations it is seen that there is a correlation between the diameter of the muscle fiber and its color, metabolite load, mitochondrial content and enzyme concentrations. Such a relationship has been shown in the pectoralis muscle of birds (George and Naik, 1957; George and Scaria, 1958a; George and Talesara, 1960, 1961), bats (George, Susheela and Scaria, 1958) and the flight muscles of insects (George and Bhakthan, 1960a, 1960b). However, in the case of the fibers of the diaphragm in which the narrowest white fiber and broadest red fiber have diameters of 49 μ and 43 μ, respectively, the above correlation was not so marked as in the case of the broad and narrow fibers of the pigeon breast muscle. In the bat pectoralis such variations in diameter were observed (George, Susheela and Scaria, 1958). The fibers of the pigeon breast muscle are therefore to be regarded as an instance of extreme specialization achieved in the differentiation of the two types of fibers.

In the diaphragm the broadest white fibers are distinctly loaded with glycogen and contain less mitochondria (as evinced by the staining for lipid) and less lipase and succinic dehydrogenase activity. In the case of the narrower white fibers and broader red fibers, however, it was not possible to arrive at definite conclusions based on histochemical observations. It also became clear that instead of the high degree of specialization resulting in the two distinct fiber types as in the pigeon breast muscle, in the diaphragm, the differentiation of the component fibers achieved a greater extent of variability in structure and perhaps function too. On the other hand, the diaphragm achieved a specialization in having three distinct regions, which is revealed from our histochemical and quantitative observations.

The fact that the dorsal region possesses the highest number of narrow fibers, high fat content, highest lipase activity and high succinic dehydrogenase activity suggests a predominance of fat metabolism involving the use of fat as fuel for muscular contraction. And it should be mentioned here that the utilization of fat by the diaphragm has been demonstrated by Wertheimer and Ben-Tor (1952). It has also been observed (George and Susheela, 1961) that during starvation there is a six-times actual reduction of fat in the dorsal as well as in the ventral regions over the lateral region, of the rat diaphragm.

The predominance of fat metabolism also appears evident in the ventral region where there is the highest fat content and high lipase activity. But this region comparatively has the lowest level of oxidative capacity in terms of succinic de-

hydrogenase activity. This indicates that in this region there is more of building up of fat from fatty acids for storage than fat metabolized for energy. Some indirect evidence for the possibility of fat being built up as energy store at sites rich in lipase activity, but poor in oxidative enzyme activity, has been recently presented by George and Iype (1960). They have demonstrated in the sheep heart high lipase activity and very low succinic dehydrogenase activity in the Bundle of His, and suggested the possibility of this site in the heart being a center for lipogenesis. They also demonstrated high concentrations of both the enzymes in the myocardium which is known to utilize fat as the major fuel for the activity of the heart. Further, the works of Cogan and Kuwabara (1957) and Kuwabara and Cogan (1960) have shown that the Purkinje fibers, the structural units of the Bundle of His, have the capacity to synthesize sudanophilic fat.

From the characteristics of the lateral region mentioned earlier, it is obvious that this region is specialized for carbohydrate metabolism in which glycogen forms the fuel for muscular contraction. The very high succinic dehydrogenase activity, even higher than what is seen in the fat-loaded dorsal and ventral regions, is certainly indicative of high oxidative metabolism involving the rapid oxidation through the Krebs cycle of the products of the Emden-Meyerhof cycle. Another possibility is that, under conditions of prolonged rapid respiratory activity, this region might well be capable of oxidizing fatty acids transported from the other regions of the diaphragm and/or from other sites in the body where fat is stored. In this context it may be mentioned that a recent study of the blood supply of the rat diaphragm (Beck and Baxter, 1960) has revealed the existence of an elaborate and copious system of circulation in this organ. Of the three regions, the lateral region has the major blood supply while the ventral has the least. Moreover, the blood supplies to the lateral and dorsal regions are more directly from the aorta through the phrenic arteries, than the ventral which receives blood from a small branch of the internal mammary artery. Their study has also shown the remarkable consistency in the main features of the venous drainage and the presence of an anastomatic system of veins in the diaphragm, connecting the inferior vena cava and azygous veins.

The present investigation on the diaphragm has revealed more of the complex nature of this organ than it has actually contributed to the elucidation of our knowledge of its structure and physiology. To that extent we feel amply rewarded. However, more extensive histophysiological and biochemical studies on the different regions are called for and are in progress.

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Summary

1. The nature and distribution of two types of fibers in the rat diaphragm, with respect to its dorsal (vertebral), lateral (costal), and the ventral (sternal) regions, have been studied.

2. Histochemical as well as quantitative studies on the fat and glycogen contents and the enzymic activity (lipase and succinic dehydrogenase) have been made in the above three regions.

3. It is concluded that the narrow red type of fibers is well adapted for aerobic metabolism, involving mainly the oxidation of fat for energy, and the other, the broad white variety, for anaerobic metabolism where glycogen is the chief fuel. The significance of the regional differences in the two types of fibers in the physiology of the diaphragm is discussed.

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